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## Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma

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**Abstract:** BACKGROUND Immune checkpoint inhibitors targeting programmed cell death 1 (PD1) or its ligand (PD-L1) showed activity in several cancer types. METHODS We performed immunohistochemistry for CD3, CD8, CD20, HLA-DR, phosphatase and tensin homolog (PTEN), PD-1, and PD-L1 and pyrosequencing for assessment of the O6-methylguanine-methyltransferase (MGMT) promoter methylation status in 135 glioblastoma specimens (117 initial resection, 18 first local recurrence). PD-L1 gene expression was analyzed in 446 cases from The Cancer Genome Atlas. RESULTS Diffuse/fibrillary PD-L1 expression of variable extent, with or without interspersed epithelioid tumor cells with membranous PD-L1 expression, was observed in 103 of 117 (88.0%) newly diagnosed and 13 of 18 (72.2%) recurrent glioblastoma specimens. Sparse-to-moderate density of tumor-infiltrating lymphocytes (TILs) was found in 85 of 117 (72.6%) specimens (CD3+ 78/117, 66.7%; CD8+ 52/117, 44.4%; CD20+ 27/117, 23.1%; PD1+ 34/117, 29.1%). PD1+ TIL density correlated positively with CD3+ ( $P < .001$ ), CD8+ ( $P < .001$ ), CD20+ TIL density ( $P < .001$ ), and PTEN expression ( $P = .035$ ). Enrichment of specimens with low PD-L1 gene expression levels was observed in the proneural and G-CIMP glioblastoma subtypes and in specimens with high PD-L1 gene expression in the mesenchymal subtype ( $P = 5.966e-10$ ). No significant differences in PD-L1 expression or TIL density between initial and recurrent glioblastoma specimens or correlation of PD-L1 expression or TIL density with patient age or outcome were evident. CONCLUSION TILs and PD-L1 expression are detectable in the majority of glioblastoma samples but are not related to outcome. Because the target is present, a clinical study with specific immune checkpoint inhibitors seems to be warranted in glioblastoma.

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## **Programmed death ligand 1 (PD-L1) expression and tumor infiltrating lymphocytes in glioblastoma**

**Running title:** PD-L1 expression in glioblastoma

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## ABSTRACT

**Background:** Immune checkpoint inhibitors targeting programmed cell death (PD) 1 or its ligand (PD-L1) showed activity in several cancer types.

**Methods:** We performed immunohistochemistry for CD3, CD8, CD20, HLA-DR, phosphatase and tensin homolog (PTEN), PD-1, and PD-L1 and pyrosequencing for assessment of the O6-methylguanine-methyltransferase (MGMT) promoter methylation status in 135 glioblastoma specimens (117 initial resection, 18 first local recurrence). PD-L1 gene expression was analyzed in 446 cases from Human Cancer Genome Atlas.

**Results:** Diffuse/fibrillary PD-L1 expression of variable extent, with or without interspersed epithelioid tumor cells with membranous PD-L1 expression, was observed in 103/117 (88.0%) newly diagnosed and 13/18 (72.2%) recurrent glioblastoma specimens. Sparse to moderate density of tumor infiltrating lymphocytes (TILs) was found in 85/117 (72.6%) specimens (CD3+ 78/117, 66.7%; CD8+ 52/117, 44.4%; CD20+ 27/177, 23.1%; PD1+ 34/117, 29.1%). PD1+ TIL density correlated positively with CD3+ ( $p<0.001$ ), CD8+ ( $p<0.001$ ), CD20+ TIL density ( $p<0.001$ ) and PTEN expression ( $p=0.035$ ). Enrichment of cases with low PD-L1 gene expression levels was observed in the proneural and G-CIMP glioblastoma subtypes and of cases with high PD-L1 gene expression in the mesenchymal subtype ( $p = 5.966e-10$ ). No significant difference of PD-L1 expression or TIL density between initial and recurrent glioblastoma specimens and no correlation of PD-L1 expression or TIL density with patient age or outcome was evident.

**Conclusion:** TILs and PD-L1 expression are detectable in the majority of glioblastoma samples, but are not related to outcome. As the target is present, a clinical study with specific immune checkpoint inhibitors seems to be warranted in glioblastoma.

Keywords: glioblastoma; immune check-point; programmed death 1; programmed death ligand 1,

## INTRODUCTION

Glioblastoma is the most common primary brain tumor of adults and is associated with high morbidity and poor median overall survival times of approximately 14 to 17 months across contemporary studies.<sup>1</sup> The current treatment standard for newly diagnosed glioblastoma comprises maximal safe resection followed by combined radiochemotherapy with temozolomide. So far, experimental treatments with a number of biologicals such as anti-invasive (e.g. cilengitide) and anti-angiogenic (e.g. cediranib, bevacizumab) agents or inhibitors of specific oncogenic signaling pathways (e.g. erlotinib, gefitinib, imatinib, temsirolimus, everolimus, enzastaurin) have failed to show overall survival benefits in glioblastoma.<sup>2-6</sup> Novel treatment concepts based on biological insights are urgently needed to improve patient outcomes.

Glioblastoma has long been recognized as an immunosuppressive neoplasm characterized by activation of various immune escape mechanisms including upregulation of transforming growth factor- $\beta$  (TGF- $\beta$ ), indoleamine 2,3 dioxygenase (IDO) and programmed death ligand 1 (PD-L1, also known as B7-H1).<sup>7-9</sup> Emerging evidence suggest differential immunogeneity of molecular glioblastoma subtypes with the mesenchymal subtype showing more prominent infiltration by tumor-associated lymphocytes (TILs) and expression of immuno-inhibitory molecules.<sup>10, 11</sup> Furthermore, loss of phosphatase and tensin homolog (PTEN), a recurring molecular alteration in glioblastoma, has been postulated to induce PD-L1 expression.<sup>9</sup>

Recently, a novel class of immune modulatory antineoplastic agents, so called immune check-point inhibitors, showed impressive activity with high response rates and durable tumor remissions in several cancer types including melanoma, lung cancer, renal cell carcinoma, bladder cancer and head and neck cancer.<sup>12, 13</sup> These agents act by blocking immunosuppressive receptors that inhibit effector T-cells and thus facilitate the anti-tumor immune response. Currently, immune check-point inhibitors targeting the programmed death 1 (PD-1) molecule and its ligand PD-L1 are in advanced clinical development and show compelling activity and favorable toxicity profiles across tumor types. PD-1 is a cell surface co-inhibitory receptor that is expressed on CD3+/CD8+ T-cells after activation and limits immune response, thus resulting in functionally “exhausted” T cells.<sup>14</sup> Importantly, tumoral expression of PD-L1 as assessed by immunohistochemistry seems to correlate with response to

PD-1/PD-L1 inhibitors and may emerge as a clinically relevant biomarker.<sup>13, 15</sup> Cumulating evidence, mostly from preclinical studies and experimental models, indicates a prominent activation of PD-L1 in glioblastoma.<sup>9, 16-22</sup> However, systematic studies on the expression of PD-L1 in human tissue samples are missing so far. We thus set out to characterize PD-L1 expression and its association with other tissue-based and clinical parameters in a well-annotated retrospective series of human glioblastoma samples including matched recurrent tumors to provide a basis for clinical trials and translational biomarker research. We additionally investigated PD-L1 gene expression and its correlation with patient outcome and molecular glioblastoma subtypes in the dataset of The Human Cancer Genome Atlas (TCGA).

## METHODS

### Patients and materials

#### Vienna retrospective cohort

We retrospectively identified formalin-fixed and paraffin-embedded (FFPE) tumor tissue specimens of 117 adult patients who underwent neurosurgical resection of newly diagnosed glioblastoma at the Department of Neurosurgery, Medical University of Vienna, between 2006 and 2012 from the Neuro-Biobank of the Institute of Neurology, Medical University of Vienna. In 18 patients an additional FFPE tumor specimen from the neurosurgical resection of the first local recurrence was available. Therefore, a total of 135 specimens (117 newly diagnosed glioblastomas, 18 matched local recurrences) were used for this study. Histological diagnosis of glioblastoma was performed according to the current WHO classification 23. Clinical data were extracted by chart review and survival data were retrieved from the Austrian Brain Tumor Registry.<sup>24, 25</sup> The ethics committee of the Medical University of Vienna approved the study (Vote 078/2004).

#### The Cancer Genome Atlas (TCGA) data set

We obtained clinical and GBM gene expression data for a cohort of 446 patients available at TCGA (<https://tcga-data.nci.nih.gov/tcga/>). Expression levels of the PDL-1 (CD274) gene were derived from the TCGA Agilent custom design platform represented by a single probe (A\_23\_P338479). The clinical data and annotation for MGMT promoter methylation and other molecular annotation were taken from Brennan et al 2013.<sup>26</sup>

### ***MGMT* pyrosequencing**

For determination of the *MGMT* promoter methylation status, six 10 µm sections of newly diagnosed glioblastoma FFPE tissue blocks from the Vienna cohort were cut into an Eppendorf tube. DNA isolation was performed using the EpiTect FFPE Lysis Kit (Qiagen) and bisulfite modification was performed using the EpiTect Fast FFPE Bisulfite Kit (Qiagen). Pyrosequencing analysis was performed on a PyroMark Q24



MDx (Qiagen, Germany) system with the theascreen MGMT Pyro Kit (Qiagen) 27. In line with previous publications, cases with a mean methylation percentage of <8% were regarded as MGMT promoter unmethylated and cases with a mean methylation percentage of  $\geq 8\%$  were regarded as MGMT promoter methylated.<sup>27, 28</sup>

### **Immunohistochemical staining**

For immunohistochemical staining, FFPE blocks from the Vienna cohort were cut into serial 3  $\mu\text{m}$  slices with a microtome. Immunohistochemistry for CD3, CD8, PD-1, CD20, HLA-DR, GFAP and PTEN was performed on a Ventana Benchmark Ultra immunostaining system (Ventana, Tucson, AZ, USA) and immunohistochemistry for PD-L1 was performed using a Dako AutostainerPlusLink immunostaining system (Dako, Glostrup, Denmark). In order to facilitate comparison of regional distributions of PD-L1 and PTEN expression and TIL infiltration, immunostaining was performed on adjacent sections. Antibodies and immunostaining protocols are listed in **Supplemental Table 1**. FFPE tissue blocks of human non-neoplastic lymph nodes (TILs) and human placenta (PD-L1) were used as positive controls. For PTEN immunostaining, endothelial cells within the tumor tissue served as internal positive controls.

### **Evaluation of immunohistochemistry**

#### PD-L1 and PTEN expression

PD-L1 expression was descriptively recorded according to the cellular and topographical localization, distribution and intensity of the immunohistochemical signal.

The extent of diffuse/fibrillary PD-L1 and PTEN expression throughout the tumor tissue was semiquantitatively assessed according to the following criteria 18, 29: (1) no positive tumor areas; (2) expression in less than 25% of non-necrotic tumor area; (3) expression in more than 25% and less than 50% of non-necrotic tumor area; (4) expression in more than 50% and less than 75% of non-necrotic tumor area; (5) expression in more than 75% of non-necrotic tumor area.

Presence of epithelioid tumor cells with distinct membranous anti-PD-L1 labelling was recorded only if they constituted more than 5% of all tumor cells by semiquantitative assessment in order to comply with the cut-off used in previous publications.<sup>13</sup>

### TIL density

Density of CD3+, CD8+, PD1+ and CD20+ TIL subsets was evaluated by overall impression at low microscopic magnification (100x). Furthermore, the densities were separately scored at higher magnification (200x - 400x) in predefined regions of interest, namely: (1) within the glioblastoma tissue; (2) in perivascular regions; and (3) in the infiltration zone to the brain parenchyma. Previously published semiquantitative evaluation criteria were used to describe TIL infiltration density and TIL density was judged to be sparse, moderate or dense.<sup>30</sup> The regional distributions of the various TIL subset infiltrates and PD-L1-positive and PTEN-positive tumor areas were compared on adjacent tissue sections.

### **Statistical analyses**

Spearman correlation was applied to analyze correlation of two ordinal parameters. Chi Square test was used to assess group differences as appropriate. Overall survival from diagnosis of glioblastoma to death or last follow up was estimated using the Kaplan Meier limit method. The log rank test was used to assess group differences. A cox regression model was used to analyze the association of continuous variables with survival. A two-tailed significance level of 0.05 was applied. Because of the exploratory and hypothesis generating design of the present study, no adjustment for multiple testing was applied 31. All statistical analysis was performed with statistical package for the social sciences (SPSS) 20.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Vienna retrospective cohort

#### Patients

The patients' characteristics including clinical baseline parameters, details on administered therapies and results of MGMT promoter methylation status testing are listed in **Table 1**.

#### PD-L1 expression

We found prominent PD-L1 staining of variable extent in glioblastoma tissues, while the surrounding CNS tissue generally showed no or only very faint and diffuse focal labelling of the neuropil and occasional light staining of single and dispersed parenchymal cells such as neurons and microglia/macrophages (**Figure 1**).

The majority of glioblastoma cases, 103/117 (88.0%) of newly diagnosed and 13/18 (72.2%) of recurrent cases, showed a prominent diffuse/fibrillary expression pattern of variable extent (**Figure 2**). Diffuse/fibrillary PD-L1 expression was typically found in a patchy distribution throughout the tumor tissue without accentuation in the perivascular or perinecrotic areas.

In 44/117 (37.6%) of newly diagnosed and 3/18 (16.7%) of recurrent glioblastomas interspersed singular or focally aggregated epithelioid tumor cells with distinct and strong membranous PD-L1 immunolabelling was found (**Figure 2**). In some of these tumors in addition to the membranous signal also some faint diffuse or granular cytoplasmic staining was observed. Epithelioid PD-L1+ tumor cells were exclusively found in cases with diffuse/fibrillary PD-L1 expression.

**Table 2** details the results of semiquantitative evaluation of tumoral PD-L1 expression.

In 48/117 (41.0%) specimens with newly diagnosed glioblastoma, surrounding brain parenchyma was present and thus evaluable for PD-L1 expression. Among these cases, we found some faint anti-PD-L1 staining of neurons in 32/48 (66.7%) cases, while in 16/48 (33.3%) specimens no neuronal PD-L1 expression was detected.

### TILs and microglia/macrophages

TIL infiltration of variable density was observed in 85/117 (72.6%) of newly diagnosed and 15/18 (83.3%) of recurrent glioblastoma specimens. TIL infiltration was in general of sparse to moderate density with TILs mostly located in perivascular areas and in zones of tumor invasion into the surrounding brain parenchyma (**Figure 3**). TILs were only infrequently found within the tumor tissue and in peri-necrotic areas. HLA-DR+ microglia/macrophages were distributed diffusely throughout the tumor tissue. **Table 3** shows the results of semiquantitative evaluation of the density and distribution of CD3+, CD8+, CD20+, PD1+ and HLA-DR+ cells.

### PTEN expression

Anti-PTEN immunostaining distinctly labelled endothelial cells. Diffuse/fibrillary and membranous PTEN expression of tumor tissue was detected to a variable extent in a patchy pattern through the tumor tissue, without a clear-cut accentuation around necrotic areas or vascular structures.

Among newly diagnosed glioblastoma specimens, the semiquantitative distribution of tumoral PTEN expression was as follows: 27/117 (23.1%) cases: negative; 33/117 (28.2%) cases: less than 25% of tumor tissue positive; 20/117 (17.1%) cases: more than 25% and less than 50% of tumor tissue positive; 22/117 (18.8%) cases: more than 50% and less than 75% of tumor tissue positive; 15/117 (12.8%) cases: more than 75% of tumor tissue positive.

Among recurrent glioblastoma specimens, the semiquantitative distribution of tumoral PTEN expression was as follows: 8/18 (44.4%) cases: negative; 2/18 (11.1%) cases: less than 25% of tumor tissue positive; 2/18 (11.1%) cases: more than 25% and less than 50% of tumor tissue positive; 4/18 (22.2%) cases: more than 50% and less than 75% of tumor tissue positive; 2/18 (11.1%) cases: more than 75% of tumor tissue positive.

### Comparison of the spatial distribution of tissue-based parameters

Comparative evaluation of the spatial distribution of the various TIL subsets on adjacent sections showed regional overlap of TIL infiltration as CD3+, CD8+ and PD1+ TILs were evident in the same areas in consecutive sections (newly diagnosed: 51/117, 43.6%; recurrent: 12/18, 66.7%). Infrequently, regional overlap of TIL infiltration and PD-L1 expression was observed (newly diagnosed: 18/117, 15.4%; recurrent: 6/18, 33.3%). No clear-cut accumulation of PD1+ TILs in the immediate proximity to PD-L1+ glioblastoma cells or PD-L1+ macrophages was observed.

In most cases, we found no regional overlap of PTEN-negative and PD-L1 positive areas on adjacent sections. Only in a minority of cases (newly diagnosed: 31/117, 26.5%; recurrent: 6/18, 33.3%), we found discordant expression profiles with PD-L1 positive areas lacking PTEN expression.

### Statistical analyses

#### *Correlation of tissue-based parameters*

PD1+ TIL density correlated positively with CD3+ TIL density ( $p < 0.001$ ; Chi Square), CD8+ TIL density ( $p < 0.001$ ; Chi Square), CD20+ TIL density ( $p < 0.001$ ; Chi Square) and PTEN expression ( $p = 0.035$ ; Chi Square test), but not with density of HLA-DR+ microglia/macrophages ( $p = 0.215$ ; Chi Square test), MGMT promoter methylation status ( $p = 0.195$ ; Chi Square test), membranous PD-L1 expression on epithelioid tumor cells ( $p = 0.808$ ; Chi Square test) or diffuse/fibrillary PD-L1 expression ( $p = 0.766$ ; Chi Square test).

Diffuse/fibrillary PD-L1 expression was not correlated with CD3+ TIL density ( $p = 0.168$ ; Chi Square test), CD8+ TIL density ( $p = 0.068$ ; Chi Square test), CD20+ TIL density ( $p = 0.516$ ; Chi Square test) or HLA-DR+ microglia/macrophages ( $p = 0.897$ ; Chi Square test), PTEN expression ( $p = 0.232$ ; Chi Square test) or MGMT promoter methylation status ( $p = 0.335$ ; Chi Square test). Furthermore, there was no correlation between presence of epithelioid tumor cells with membranous PD-L1 expression and CD3+ TIL density ( $p = 0.555$ ; Chi Square test), CD8+ TIL density ( $p = 0.380$ ; Chi Square test), CD20+ TIL density ( $p = 0.093$ ; Chi Square test), HLA-DR+

microglia/macrophages ( $p=0.512$ ; Chi square test), PTEN expression ( $p=0.329$ ; Chi Square test) or MGMT promoter methylation status ( $0.181$ ; Chi square test).

#### *Correlation of tissue-based parameters with clinical characteristics*

There was no correlation between pre-surgical treatment with corticosteroids and membranous PD-L1 expression ( $p=0.283$ ; Chi Square test), diffuse/fibrillary PD-L1 expression ( $p=0.513$ ; Chi Square test), density of PD1+ TILs ( $p=0.534$ ; Chi Square test), CD3+ TILs ( $p=0.889$ ; Chi Square test), CD8+ TILs ( $p=0.190$ ; Chi Square test), CD20+ TILs ( $p=0.917$ ; Chi Square test) or density of HLA-DR+ microglia/macrophages ( $p=0.542$ ; Chi Square test). Further, no correlation of age ( $< 65$  years vs.  $> 65$  years) and membranous PD-L1 expression ( $p=0.612$ ; Chi Square test), diffuse/fibrillary PD-L1 expression ( $p=0.383$ ; Chi Square test), density of PD1+ TILs ( $p=0.945$ ; Chi Square test), density of CD3+ TILs ( $p=0.418$ ; Chi Square test), density of CD8+ TILs ( $p=0.376$ ; Chi Square test) or density of HLA-DR microglia/macrophages ( $p=0.758$ ; Chi Square test) was evident. However, younger patients ( $< 65$  years) presented more frequently with sparse and moderate density of CD20+ TILs than patients aged  $\geq 65$  years at diagnosis of GBM ( $p=0.043$ ; Chi Square test).

#### *Comparison between specimens of first and second resection*

The extent of diffuse/fibrillary PD-L1 expression between newly diagnosed and recurrent glioblastoma specimens was not different ( $p=0.411$ ; Chi Square test; **Table 4**). Epithelioid tumor cells with anti-PD-L1 membrane labelling were more common in initial tumors (9/18; 50%) than in recurrent tumors (3/18; 16.7%;  $p=0.034$ ; Chi Square test).

There were no differences between first and second resection for PD1+ TIL density ( $p=0.070$ ; Chi Square test), CD3+ TIL density ( $p=0.237$ ; Chi Square test), CD8+ TIL density ( $p=0.103$ ; Chi Square test), CD20+ TIL density ( $p=0.122$ ; Chi Square test), HLA-DR+ microglia/macrophage density ( $p=0.085$ ; Chi Square test) or PTEN expression ( $p=0.289$ ; Chi Square test).

### *Survival analyses*

Young patient age at diagnosis of glioblastoma (< 65 years: 16 months vs.  $\geq$  65 years 11 months;  $p=0.034$ ; log rank test), high KPS (< 70: 4 months vs.  $> 70$ : 17 months;  $p<0.001$ ; log rank test), greater extent of resection (subtotal: 12 months vs. total: 18 months;  $p=0.003$ ; log rank test) and MGMT promoter hypermethylation (< 8%: 11 months vs.  $> 8\%$ : 18 months;  $p=0.002$ ; log rank test) correlated with overall survival, while density of PD1+ TILs (absent: 15 months vs. present 15 months;  $p=0.981$ ; log rank test), presence of diffuse/fibrillary PD-L1 expression (absent: 15 months vs. present 15 months;  $p=0.921$ ; log rank test) and presence of epithelioid tumor cells with membranous PD-L1 expression (< 5%: 15 months vs.  $> 5\%$  14 months;  $p=0.724$ ; log rank test), or presence of PD-L1- neurons (negative 15 months vs. positive 12 months;  $p=0.533$ ; log rank test) showed no impact on survival times (**Figure 4**).

### **TCGA data-set**

#### Correlation of PD-L1 gene expression with molecular glioblastoma subtypes

In order to see if there was an association between PD-L1 expression levels and known expression subtypes, we classified samples into PD-L1 high/low based on median PD-L1 expression levels. The results in **Table 5** showed that there is a statistically significant difference in the distribution of expression subtypes between PD-L1 high and low samples ( $p\text{-value} = 5.966\text{e-}10$ , Pearson's Chi-squared test), with a particularly evident enrichment of proneural and G-CIMP expression subtypes in the PD-L1 low group, while the mesenchymal expression subtype is over-represented in the PD-L1 high group.

#### Survival analyses

Survival analysis using Cox proportional hazard (CPH) regression was performed in two separate subsets of the data. The first subset comprised a total of 446 patients. As the MGMT promoter methylation status was not complete for the 446 patients, it was excluded as a covariate, and only age and PD-L1 expression levels were included. Both in the univariate and multivariable CPH regression, PD-L1 had no

statistically significant association with overall survival of patients (**Figure 4H, Table 6**).



## DISCUSSION

PD/PD-L1 interactions are now considered central to the immunological control of cancer. Here we show prominent expression of PD-L1 by tumor cells in a majority human glioblastoma samples. We observed two main staining patterns, which likely relate to the particular and heterogeneous microarchitecture of glioblastoma that is captured in the widely used descriptive addendum “multiforme”. First, we detected clear membranous PD-L1 expression with distinct highlighting of tumor cell surfaces, as is seen in epithelial cancers and melanoma.<sup>15</sup> This expression pattern was mainly found on roundish, non-fibrillar glioblastoma cells with well-delineated cytoplasm and cell membranes that have some histological similarities with epithelial cells. Such “epithelioid” tumor cells are a well-recognized histological feature of glioblastomas and in our series were interspersed within the tumor tissue as single cells or focal tumor cell aggregates in 37.6% of newly diagnosed and 16.7% of recurrent glioblastoma specimens.<sup>23, 32</sup> Second, in some of these tumor cells, we observed in addition to the membranous signal also some faint diffuse or granular cytoplasmic staining. This may relate to internalized surface PD-L1 molecules, as PD-L1 storage and degradation in lysosomes has been described in lymphoma models.<sup>33</sup> In many cases (88.0% of newly diagnosed and 72.2% of recurrent glioblastoma cases), a prominent diffuse/fibrillary PD-L1 immunostaining pattern of variable extent was seen throughout the tumor tissue. Due to the intensity of this staining pattern, we consider it very likely that this relates to membrane-bound PD-L1 on the delicate and intermingled tumor cell processes that form the pathognomic “neurofibrillary matrix” of diffuse astrocytic gliomas. However, distinct labelling of tumor cell membranes cannot be appreciated in this particular histological presentation due to the limited spatial resolution at the light-microscopic level. We did not have the opportunity to investigate the subcellular distribution of PD-L1, e.g. by transmission electron microscopy with immuno-labelled particles. Notably, we observed no or only very faint focal and diffuse staining in the neuropil in tumor-surrounding CNS tissue parts with occasional light labeling of parenchymal cell elements. Glial cells (astrocytes, microglia, oligodendrocytes) and neurons have been shown to express low or undetectable PD-L1 under basal conditions, but are able to up-regulate PD-L1 in response to cytokine release in neuroinflammatory processes.<sup>34, 35</sup>

The rate of PD-L1-positive cases in glioblastoma found in our study seems high in comparison to other solid tumor types, where approximately 30% of melanoma cases and 25 to 36% of non-small cell lung cancer cases have been found to express PD-L1 by immunohistochemistry.<sup>36, 37</sup> Our findings clearly corroborate previous studies in experimental models, suggesting a prominent involvement of the PD-1/PD-L1 axis in creating an immunosuppressive microenvironment in glioblastoma and taken together our data and the experimental evidence provide a strong rationale for clinical trials investigating immune check-point inhibitors in this indication. Of particular interest in this context are recent reports showing durable therapeutic efficacy of immune check-point inhibition in animal models of glial brain tumors.<sup>8, 22</sup> The high prevalence also allows clinical studies without pre-testing the PD-L1 status in the tumor tissue, although *post hoc* correlation of the extent of PD-L1 expression with response to treatment may be of interest. Of note, therapeutic efficacy of the CTLA4 antibody ipilimumab against melanoma brain metastases has been documented, thus showing the feasibility of antibody-mediated immune-checkpoint inhibition in intraparenchymal CNS lesions.<sup>38, 39</sup> However, further studies will be important to investigate in detail the role of the blood-brain/blood-tumor barrier in drug penetration and intracerebral efficacy of immune check-point inhibitors. Given this uncertainty and the availability of PD1/PD-L1 inhibitors, these may also an option for trials in patients with gliomas.

TIL infiltrates were in general sparse with some concentration of in the perivascular areas rather than in the tumor tissue. This finding is well in line with previous observations on the distribution of TILs in glioblastoma and suggests that TILs might not be able to readily migrate into the immunosuppressive tumor microenvironment and arrest in the perivascular.<sup>40, 41</sup>

Comparative analysis of newly diagnosed and 18 matched recurrent glioblastomas showed no clear up- or down-regulation of PD-L1 and no significant changes in the density of PD1+, CD3+, CD8+, or CD20+ TILs over time. Diffuse/fibrillary PD-L1 expression was common in both newly diagnosed and recurrent specimens. Interspersed epithelioid cells with distinct membranous PD-L1 staining were more commonly found in newly diagnosed glioblastoma specimens than in recurrent cases. However, this finding may be influenced by sampling error and does not necessarily indicate a clinically relevant dynamic in PD-L1 expression.

We used in our study the non-commercial anti-PD-L1 antibody 5H1, which was kindly provided by Dr. Lieping Chen (Yale University). Specificity of this antibody was confirmed in a recent publication, while some commercially available antibodies failed to show reliable PD-L1 labelling.<sup>36</sup> Using the 5H1 antibody, a correlation of immunohistochemically detected PD-L1 expression with response to PD1/PD-L1 inhibition has been shown in metastatic melanoma.<sup>13, 15, 42</sup> However, responses to PD-1/PD-L1 inhibitors are observed in a significant fraction of patients with PD-L1 negative tumor samples and the true predictive value of PD-L1 expression is under intense investigation in ongoing clinical trials.<sup>15, 37</sup>

An association of PD-L1 expression and PTEN loss via PI3K/AKT pathway signaling has been shown in several studies in squamous cell cancer, triple-negative breast cancer, pancreatic cancer, colorectal cancer, and glioblastoma.<sup>9, 18, 20, 43-45</sup> However, in our study on human glioblastoma samples we did not find a correlation of PD-L1 expression and lack of PTEN expression at the protein level and this lack of correlation is consistent with a recent study on melanoma.<sup>46</sup> PTEN protein expression has been shown to poorly correlate with (epi-)genetic PTEN aberrations in glioblastoma and further studies may address whether PTEN methylation, PTEN mutations, or PTEN deletions correlate with PD-L1 expression in glioblastoma tissue samples.<sup>29</sup>

Interestingly, we found we found a highly significant association of PD-L1 gene expression with molecular glioblastoma subtypes with an enrichment of PD-L1 low cases in the proneural and G-CIMP subtypes and PD-L1 high cases in the mesenchymal subtype. These results are well in line with some previous studies showing evidence for increased immunologic activity in mesenchymal glioblastomas and may be explained by an interaction of PD-L1 with signal transducer and activator of transcription 3 (STAT3), as STAT3 has been shown to be a major promoter of PD-L1 expression and to be up-regulated in mesenchymal glioblastomas.<sup>11, 47-50</sup> In any case, based on our findings clinical studies with PD1/PD-L1 inhibitors need to include investigation of differential response patterns across molecular glioblastoma subtypes.

Studies on the prognostic impact of tumoral PD-L1 expression have shown inconsistent results across tumor types.<sup>20, 51-53</sup> In our series including a total of 563 glioblastoma cases from two independent cohorts, there was no association of PD-L1

expression with patient outcome. In particular, the prognostic impact of neuronal PD-L1 protein expression recently described in a very small series of 17 glioblastoma cases was not corroborated in our series of 117 Viennese cases.<sup>19</sup> We appreciate that the power of survival analyses in our series is limited by the sample size and the retrospective study design and our results surely need confirmation in larger and prospectively collected cohorts. However, the known prognostic factors patient age, Karnofsky performance status, extent of resection, and MGMT promoter methylation status showed the expected separation of prognostic groups and thus validate the suitability of our cohort for exploratory survival analyses.

In conclusion, this analysis shows that PD1 and/or PD-L1 are immunohistochemically detectable in a majority of glioblastoma samples, thus suggesting that the immunosuppressive PD1/PD-L1 axis is active in glioblastoma. A clinical study with specific immune checkpoint inhibitors is warranted in glioblastoma and it remains to be seen whether PD-L1 expression patterns or molecular glioblastoma subtypes correlate with response to such treatments.

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## FIGURE LEGEND

**Figure 1:** Low-magnification overview (anti-PD-L1 immunostaining, original magnification x 2) showing prominent and patchy PD-L1 expression (arrow 1: positive area, arrow 2: negative area) in tumor tissue of a glioblastoma and lack of PD-L1 expression in the adjacent brain parenchyma (asterisk).

**Figure 2:** Diffuse/fibrillary and membranous PD-L1 expression in glioblastoma.

A-D (adjacent sections of the same tissue specimen): In glioblastoma areas with diffuse/fibrillary histomorphology (A: Hematoxylin and Eosin staining, original magnification x 200) and expression of glial fibrillary acidic protein (GFAP; B: anti-GFAP, original magnification x 200) we found prominent diffuse/fibrillary PD-L1 expression (C and D: anti-PD-L1; C: original magnification x 200, D: original magnification x 400).

E-H (adjacent sections of the same tissue specimen): In glioblastoma areas with epithelioid tumor cells (E: Hematoxylin and Eosin staining, original magnification x 200) and expression of glial fibrillary acidic protein (GFAP; F: anti-GFAP, original magnification x 200) we found prominent membranous PD-L1 expression and some faint diffuse or granular cytoplasmic staining (G and H: anti-PD-L1; G: original magnification x 200, H: original magnification x 400).

**Figure 3:** A: Dense CD3<sup>+</sup> TIL infiltration (magnification x 200) B: PD1<sup>+</sup> TILs infiltrating glioblastoma (magnification x 400); C: Perivascular infiltration with CD3<sup>+</sup> TILs (magnification x 200); D: CD3<sup>+</sup> TILs at the infiltration zone (magnification x 200)

**Figure 4:**

A- G: Kaplan-Meier curves in the Vienna retrospective cohort. A: Overall survival according to age; B: Overall survival according to Karnofsky performance status; C: Overall survival according to extent of resection; D: Overall survival according to MGMT methylation status; E: Overall survival according to membranous PD-L1 expression; F: Overall survival according to diffuse/fibrillary PD-L1 expression; G:

Overall survival according to PD-L1 expression in neuron. All p-values are according to the log rank test; H: Kaplan-Meier curve in the TCGA cohort showing overall survival according to PD-L1 gene expression.